AD_____

NOVEL TESTS FOR RAPID DETECTION OF INSECTICIDE RESISTANCE IN MOSQUITO VECTORS

Annual Report

George P. Georghiou, Ph.D

January 25, 1989

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5170

University of California
Department of Entomology
Division of Toxicology and Physiology
Riverside, CA 92521

DOD DISTRIBUTION STATEMENT

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclasssified	1b RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION University of California Department of Entomology 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) Division of Toxicology and Physiology Riverside, CA 92521	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING / SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER Contract No. DAMD17-85-C-5170
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 63750A; PROJECT NO. 3M4 63750D808; TASK NO. AB; WORK UNIT ACCESSION NO. 028

11. TITLE (Include Security Classification)

Novel Tests for Rapid Detection of Insecticide Resistance in Mosquito Vectors

12. PERSONAL AUTHOR(S)

George P. Georghiou, Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 6/15/87 TO 10/30/88	1989 January 25	45

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	06		RA I; Resistance; Insecticides; Mosquitoes; Resistance Mechanisms; Esterases; Acetylcholinesterase; Gene Amplification; Resistance Detection; Test Methods
06	03		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

During the third year of this contract, research emphasis was placed on the improvement and field evaluation of a practical test kit for the detection and monitoring of highly active esterases that are responsible for organophosphate insecticide resistance in mosquitoes. The evaluation of the test kit was performed on 40 field populations of mosquitoes in eight abatement districts in California and one in New Mexico. The results demonstrated the usefulness of the test kit in detecting *Culex* mosquitoes that are resistant to organophosphates due to the presence of highly active esterases.

Other research has provided information on the geographical distribution of various highly active esterases in the U.S. and certain foreign countries, and on the classical and molecular genetics of these esterases.

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Mrs. Virginia M. Miller	22b TELEPHONE (Include Area Code) 301/663-7325 22c. OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

N.A. Where copyrighted material is quoted, permission has been obtained to use such material.

N.A. Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

GAG Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

GAG In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

GAG For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

GAG In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Accession For	
NTIS GRA&I	X
DTIC TAB	☐
Unannounced	☐
Justification	
By	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A-1	

George D. Geoghegan 1-25-89
PI Signature Date

TABLE OF CONTENTS

1. OBJECTIVES

Effective management of insecticide resistance in pest populations that are under chemical control relies in large part on the development of simple tests to detect the presence of specific resistance genes (or mechanisms) in individual insects and to monitor their frequencies through time and space. During the current year emphasis was placed on the following objectives: (1) Research on non-specific esterases and field evaluation of a test kit for detection of organophosphate-detoxifying esterases and organophosphate (OP) resistance; (2) Research on insensitive acetylcholinesterase (AChE) and refinement of a diagnostic test for detection of insensitive acetylcholinesterase and OP/carbamate resistance; and (3) Further studies on other mechanisms of resistance aimed at improving the prospects for developing other diagnostic tests.

2. "NONSPECIFIC" ESTERASES:

2.1 *Influence of mosquito age or exposure to insecticides on esterase activity*

Studies on the variation of "non-specific" esterase activity (i.e. activity toward naphthyl acetate) as influenced by aging of mosquitoes or exposure to insecticides were continued. Aging was found to be associated with an important loss of esterase activity in both male and female mosquitoes (40% by day 28) (Fig. 1). However, the esterase activity remaining in such mosquitoes was still considerably higher than that found in young, susceptible insects.

The effect of mosquito contact with OP insecticides on the level of esterase activity was determined by exposing 2-day old adults for one hour to WHO filter papers impregnated with 1% fenitrothion, homogenizing mosquitoes at various time intervals thereafter, and measuring esterase activity. No significant suppression of esterase activity was observed in these mosquitoes immediately after exposure or during the subsequent 96 hours (Fig. 2). However, studies of *in vitro* inhibition of esterase activity by fenitrothion in homogenates of resistant larvae over a 10-minute period showed that inhibition was complete at a fenitrothion concentration of 0.25% (Fig. 3).

2.2 *Role of esterases A2B2 and A4B4 in resistance*

Research on the role of highly active esterases A2 and B2 in OP resistance and on their linkage relationships to the genes encoding esterases A1 and B1 was completed (publ. No. 15). Research is currently under way on the genetics of the newly discovered esterases A4 and B4 in strains of *C. p. pipiens* from Cyprus, Corsica and mainland France (and possible also Tennessee). The resistance of the Cyprus strain containing these esterases has increased from 8x to 16x (at LC_{50}) during four generations of selection with temephos.

2.3 *Geographic distribution of esterases*

Field surveys to determine the geographic distribution of various esterases involved in OP resistance were continued. Samples of *C. p. pipiens* and *C. p. quinquefasciatus* collected from various states and foreign countries were examined (a) by electrophoresis for types of esterases present, (b) by microtiter plate assay and/or FP/Est for total esterase activity, and in most cases (c) by bioassay with temephos, chlorpyrifos, fenthion and malathion (Table 1).

2.4 *Research on molecular genetics of esterases*

The increased activity of esterases B2 (in *C. p. quinquefasciatus* and *C. p. pipiens*), and B3 (in *C. tarsalis*) was shown to be due to overproduction of these esterases resulting from gene amplification (publ. No. 17), as had been found earlier with esterase B1 in *C. p. quinquefasciatus* (Mouchès et al. 1986, Science 233:778). The amplification levels of B esterases were determined to be between 18- and 512-fold the level found in susceptible strains. The variability of "amplification units" encompassing esterase B genes was analyzed by restriction enzyme digestions: large differences were found between the amplification units of different esterase B genes, but almost none between those of esterase B2 genes from North America, Asia or Africa. (This study was conducted collaboratively with Dr. N. Sivasubramanian, University of California, Riverside, and laboratories at Montpellier (Dr. N. Pasteur) and Antibes (Drs. J. Bergé and C. Mouchès), France.

2.5 *Variation of high esterase activity in populations*

The variation of esterase activity levels among individuals of a highly resistant strain (Tem-R with esterase B1), a susceptible strain (S-Lab) and in progeny of their reciprocal crosses (F_1(SL), F_1(TR)) was investigated in two separate experiments (publ. No. 18). The esterase activity distributions of the strains are shown in Figs. 4, 5, and 6. The Tem-R strain exhibited 126 and 111 times the esterase activity of the S-Lab strain in experiments 1 and 2, respectively (Table 2). The relationship between Tem-R and the F_1 strains was fairly consistent over experiments, with the F_1 strains exhibiting between 59 and 63 percent of the activity of Tem-R (Table 2).

In both experiments, the mean esterase activity of S-Lab females was significantly higher than that of S-Lab males. While female mean esterase activity was higher than that for males in F_1 strain comparisons, none of the differences was statistically significant. In contrast to the other strains, Tem-R males had higher esterase activity than females in both experiments, the difference being significant or close to significant in both experiments.

Establishing the functional relationship between esterase activity and resistance will lead to better predictive models of how amplified esterase B1 genes respond to insecticide selection in natural populations. The Tem-R strain exhibited about 120-fold the esterase activity of the S-Lab strain, yet its resistance ratio at the LC_{50} was about 590 (Table 3). This could be due to the contribution of a number of esterases to the activity measure of the S-Lab strain, some of which are not involved in insecticide detoxification, while the activity of the Tem-R strain is overwhelmingly due to the B1 esterase.

Using resistance ratios for comparison, the Tem-R strain showed about 7.8x the level of resistance of the F_1 strains (Table 3). This compares to a 1.6x difference in esterase activity between the F_1 strains and Tem-R (Table 2). This difference may be a matter of the scale on which resistance is measured. When resistance is represented on a log scale, as the log of the resistance ratio, the Tem-R strain exhibits about 1.5x the resistance of the F1 strains, a much closer fit to the observed 1.6x difference in esterase activity.

2.6 *Stability of high esterase activity in laboratory populations*

To determine the effect of relaxation of selection on generation to generation changes in esterase activity and resistance, the highly resistant strain, Tem-R, was reared for one generation (P) without its usual selection with the organophosphate insecticide temephos. The strain was split into two lines, A and B, which were reared separately without selection for five additional generations (F_1-F_5).

Resistance fluctuated somewhat from generation to generation in both line A and line B (Figs. 7 and 8). However, no general decline in resistance was observed in either cage over the five generations of the study. Mean esterase activity also fluctuated over generations (Figs. 9 and 10), again with no detectable trend toward lower mean esterase activity. In addition, there was no indication that the range of esterase activities was expanding as generations progressed (Figs. 9 and 10).

These results suggest that the amplification of B1 esterase genes is relatively stable genetically under the optimal conditions of rearing employed in this study. That is, there is no apparent tendency for genetic processes to reduce the mean copy number of esterase B1 genes in gametes.

3. TEST PROCEDURES FOR HIGHLY ACTIVE ESTERASE DETECTION

3.1 *Filter paper test (FP/Est test)*

A filter paper test (FP/Est test) for detecting the presence of esterases that are involved in OP resistance, based on the method of Pasteur and Georghiou (1981, Mosq. News 41:181) was improved further for use under practical field conditions (publ. No. 13). The method is based on the deposition of mosquito homogenates on filter paper which is subsequently incubated in buffer containing alpha-naphthyl acetate and then immersed in a staining solution containing Fast Garnet GBC to reveal the esterase degradation products. This improved procedure permits clear discrimination of increased esterase activity in single mosquitoes by visual inspection as well as by densitometric analysis.

Validation tests which were performed in the laboratory on several field collections of mosquitoes confirmed that the proportion of insects with susceptible-like esterase activity observed by means of the FP/Est test is strongly correlated with the proportion found to be susceptible by standard bioassays with chlorpyrifos, temephos, fenthion and malathion (publ. No. 13).

3.2 *Development of a field test kit for highly active esterases*

In view of the favorable results obtained with the FP/Est method, a prototype test kit based on this procedure was formulated for use under field conditions (see Appendix No. 1 for description of kit and procedure). After intensive testing in the laboratory, samples of the kit were distributed to entomologists in nine Mosquito Abatement Districts (eight in California, and one in New Mexico) for field evaluation. To ensure correct use of the kit, a training workshop was organized (June 10, 1988). In addition to receiving test kits, the collaborators also received written detailed procedures, forms for reporting results, and solutions of temephos, chlorpyrifos, fenthion, malathion, permethrin, and propoxur of the appropriate concentrations for diagnostic bioassay tests. The report forms and test papers were returned to us for

analysis and evaluation. Results were received from 8 collaborators. One test kit had been exposed accidentally to high temperatures in an automobile and was discarded. In all, the test kits were used against 40 larval collections (Table 4).

Tests were conducted mostly on *C. p. quinquefasciatus* and *C. p. pipiens* larvae (25 collections), but a smaller number of tests were made on *C. tarsalis*, *C. peus*, and to a limited extent on *Psorophora colombiae* and *Aedes nigromaculis*. The results of "visual" interpretation of the filter paper tests by M.A.D. entomologists were compared to densitometric evaluation of the filter papers in our laboratory.

In our earlier tests by the FP/Est method using adults of the *Culex pipiens* complex, it was determined that the threshold optical density (OD) which discriminates between mosquitoes with a highly active esterase and those in which such esterases are of low activity or absent, is OD = 0.10 (publ. No. 13). In the present tests on larvae, a consistent increase in esterase activity was noted from the 3rd to the late 4th instar in susceptible strains, most probably related to the increase in larval size during the developmental period. When data from all instars were pooled, it was concluded that the ODs that discriminate between insects with susceptible-like and resistant-like esterase activities in *C. pipiens* larvae lay between 0.15 and 0.20.

In tests involving the field collections, the proportions of insects with OD values below 0.15 and below 0.20 were overall very similar (Table 5). Only in 3 of the 25 collections of *C. pipiens*, these values were significantly different (P = 0.05). Visual interpretations by field entomologists were in general related to the densitometric measures, i.e. entomologists interpreted as S (= insects with susceptible-like esterase activity) the spots with the lowest intensities. However, as shown by the sample data from San Mateo, they had difficulty in deciding on the "threshold" staining intensity which distinguishes between susceptible-like and higher esterase activities (Fig. 11). This difficulty may be overcome by including a color chart in the FP/Est test kit.

In *C. p. quinquefasciatus* and *C. p. pipiens*, the relation between the proportion of insects with susceptible-like esterase activity was in most cases consistent with the mortality obtained at discriminating doses of insecticides (i.e. doses that are lethal to susceptible but not to resistant individuals). Bioassays at discriminating doses were conducted by the field entomologists on 16 of the 25 *C. pipiens* collections, but only 15 were considered (collection WV-2 was eliminated as it contained both *C. pipiens* and *C. peus*). Mortalities at discriminating doses of temephos, chlorpyrifos, fenthion and malathion (Table 6) were not significantly different from one another in the 5 collections which were very susceptible to the four OPs tested (San Mateo-4, Butte-1, -2, -3 and WV-1). In other collections large variations were observed in the mortalities induced by the different OPs. No particular pattern could be detected in these variations, and they may be due to a variety of factors, such as differences in the resistance spectra related or not to histories of insecticide exposures, bioassay procedures, etc.

Highly active esterases were absent from the small samples of *C. peus* and *Ps. colombiae* that were tested, but were present in the samples of *C. tarsalis* and *Ae. nigromaculis*.

Field entomologists provided useful comments which prompted further research toward improvement of the kit. Thus we investigated the effect of delays in the time between preparation of homogenate and its deposition on filter paper. No significant loss of activity was observed during waiting periods of up to 40 minutes (Fig. 12). However, delaying the processing of the filters after the deposits had been made

resulted in complete loss of esterase activity within 10 minutes, i.e. while the deposits were drying (Fig. 13). Provision for these findings was added in the test protocol.

Conclusions and future plans: Field evaluation of the FP/Est test kit by personnel of mosquito control agencies has confirmed our laboratory results by showing the usefulness of the test in (a) detecting *Culex* mosquitoes that are resistant to OP insecticides due to increased activity of detoxifying esterases, and (b) determining the frequency of resistant individuals in a population. It is now essential to distribute and test the kit more widely, both in the U.S. and overseas in order to determine its applicability under the widest variety conditions.

4. INSENSITIVE ACETYLCHOLINESTERASE AND OP RESISTANCE

4.1 *Research on insensitive acetylcholinesterase*

A strain of *C. p. pipiens* isolated from a collection made in Cyprus, contained an insensitive AChE. Preliminary results with this strain have shown the presence of inhibitory properties that are different from those observed in a strain (MSE) from France. The Cyprus strain is being selected by propoxur to maximize its resistance. Comparative studies with these two strains (Cyprus and MSE) could provide basic information on the patterns of cross-resistance that are associated with different insensitive acetylcholinesterases.

Work toward cloning the AChE-R gene is contemplated as an essential step for better understanding the role of this important enzyme in OP and carbamate resistance. We are now producing and deep-freezing large numbers of *C. p. quinquefasciatus* and *C. p. pipiens* in order to purify at least 250 µg of enzyme which will be used to obtain a specific antibody.

4.2 *Development of a test for insensitive AChE*

During the current year, we have used extensively the microtitration assay test for AChE (MT/AChE test, see 1987 Annual Report) in order to search for cases of reduced AChE sensitivity, and hence of OP and carbamate resistance, in populations of *C. p. pipiens* and *C. p. quinquefasciatus* in the U.S. and other countries. The test involves comparing AChE activity in four aliquots from single mosquito homogenates in microtiter plates (a) under "normal" conditions, (b) in the presence of a model inhibitor of AChE, and (c, d) in the presence of a carbamate (propoxur) or an organophosphate (paraoxon) at doses which inhibit sensitive AChE but have little or no effect on insensitive AChE.

No reduction in sensitivity of AChE has been selected in U.S. populations of *C. p. quinquefasciatus*, *C. p. pipiens* or *C. tarsalis* as indicated by tests on several collections from California and other states (Table 7). We have found insensitive AChE in *C. p. pipiens* populations collected in Cyprus and Greece. Insensitive AChE appears to be a common mechanism of OP resistance in European *C. pipiens* populations. It is also widespread in the malaria vector *Anopheles albimanus* in Central America and to a lesser extent in certain other *Anopheles* species elsewhere. Thus, the possibility of such mechanism of resistance appearing in the U.S. mosquitoes cannot be excluded.

The principles of the microtiter AChE test referred above have been incorporated into a practical field test (NC/AChE test) on which we have reported in 1987. Further

simplifications have been made to this test including the use of a single insecticide (a carbamate, because of safety considerations) and visual assessment of the results. A practical field kit for testing in countries where insensitive AChE is present (e.g. Central America) is being formulated.

In order to determine whether homogenates of single mosquitoes can be used to test both for insensitive AChE and for the level of detoxifying esterase, the distribution of these enzymes in different parts of the insect body was investigated. Abdomens of *Culex p. quinquefasciatus*, were found to contain 50% to 57% of the insect's total esterase activity, whereas the head and thorax combined contained 79% to 88% of total AChE activity (Fig. 14). These results suggest that these different body parts can be used, respectively, for esterase and AChE tests.

5. MALATHION-CARBOXYLESTERASE

A strain of *C. p. quinquefasciatus* obtained from Guatemala shows 29x and 7x resistance to malathion and phenthoate, respectively, but no significant resistance to other organophosphates (temephos, chlorpyrifos and fenthion) or to propoxur and permethrin. This resistance is completely synergized by the esterase inhibitor DEF, but the strain does not manifest the presence of an esterase of high activity as is found in strains with broad spectrum OP resistance. We suspect that the strain's resistance to malathion is due to "malathion-carboxylesterase" and we are currently selecting it with malathion in order to analyze its resistance characteristics and to use it as a reference strain for the development of a malathion-carboxylesterase diagnostic test.

6. GLUTATHIONE-S-TRANSFERASES (GSH) AND MIXED FUNCTION OXIDASES (MFO)

Attempts to formulate tests for the presence of GSH- or MFO-mediated enzyme activity in resistance mosquitoes have not been satisfactory. It appears that the development of practical detection tests for these enzymes must await the availability of antibodies or DNA probes. Since very large quantities of mosquitoes are needed for this type of work, preliminary investigations have been undertaken using *Musca domestica*. Presently, emphasis is placed on GSH, of which two proteins have been purified by our collaborators in France (laboratories of CNRS at Montpellier and INRA at Antibes) from fly strains provided by our laboratory. Antibodies will be prepared against these proteins and a cDNA bank subsequently isolated. These will be tested against susceptible and resistant mosquitoes. It must be pointed out, however, that laboratory tests so far suggest that the role of GSH in mosquito resistance may be of secondary importance, and that MFO's are of some significance mainly in pyrethroid resistance.

7. MECHANISMS OF OP RESISTANCE IN *AEDES AEGYPTI*

A strain of *Aedes aegypti* obtained from Tortola, Virgin Islands, in the course of a recent survey, was found to possess high (41x) resistance to temephos (publ. No. 11). The strain was selected further by temephos pressure in the laboratory during 9 generations and its resistance was raised to 182x. This resistance was found to be suppressible by DEF, suggesting the involvement of detoxifying esterases. Esterase activity has increased under selection by temephos, as shown by MT/Est tests. However, electrophoresis shows only slightly higher esterase activity in the resistant

relative to a susceptible (Rock) strain. Tests are under way to determine whether in addition to esterases, other mechanisms may also be involved.

PUBLICATIONS

*1 Mouchès, C., Fournier, D., Raymond, M., Magnin, M., Bergé, J. B., Pasteur, N. and Georghiou, G. P.
1985. Association entre l'amplification de séquences d'ADN, l'augmentation quantitative d'estérase et la résistance à des insecticides organophorés chez des moustiques du complexe *Culex pipiens*, avec une note sur une amplification similaire chez *Musca domestic*a L. C. R. Acad. Sci. Paris, 301, Sér. III:695-700.

2. Mellon, R. B.
1985. The use of chlorpyrifos and *Bacillus thuringiensis* var. israelensis in rotation to delay the development of resistance to chlorpyrifos in the southern house mosquito, *Culex quinquefasciatus* Say. M.S. thesis, Univ. of Calif., Riverside, 143 pp.

3. Raymond, M.
1986. La résistance aux insecticides organophosphorés dans le complexe *Culex pipiens* L.: Identification des mécanismes, génétique et évolution. Thèse de Doctorat, Université de Montpellier II.

*4. Mouchès, C., Pasteur, N., Bergé, J.-B., Hyrien, O., Raymond, M., Robert de Saint Vincent, B., de Sylvestri, M. and Georghiou, G. P.
1986. Amplification of an esterase gene is responsible for insecticide resistance in a California *Culex* mosquito. Science 233:778-780.

*5. Fournier, D., Bride, J. M., Mouchès, C., Raymond, M., Magnin, M. Bergé, J. B., Pasteur, N. and Georghiou, G. P.
1987. Biochemical characterization of the esterases A1 and B1 associated with organophosphate resistance in the *Culex pipiens* L. complex. Pestic. Biochem. Physiol. 27:211-217.

*6. Mouchès, C., Magnin, M., Bergé, J.-B., de Sylvestri, M., Beyssat, V., Pasteur, N. and Georghiou, G. P.
1987. Overproduction of detoxifying esterases in organophosphate-resistant *Culex* mosquitoes and their presence in other insects. Proc. Natl. Acad. Sci. 84:2113-2116.

*7. Raymond, M., Pasteur, N., Georghiou, G. P., Mellon, R. B., Wirth, M. C. and Hawley, M. K.
1987. Detoxication esterases new to California, USA, in organophosphate-resistant *Culex quinquefasciatus* (Diptera: Culicidae). J. Med. Entomol. 1:240-27.

---

* This list includes all papers published, in press, or submitted, pertaining to research under contract DAMD17-85-C-5170. Papers indicated by asterisk contain acknowledgement of support under this contract.

*8. Raymond, M., Pasteur, N. and Georghiou, G. P.
1987. Inheritance of chlorpyrifos resistance in *Culex pipiens* L. (Diptera: Culicidae) and estimation of the number of genes involved. Heredity 58:351-356.

*9. Prabhaker, N., Georghiou, G. P. and Pasteur, N.
1987. Genetic association between highly active esterases and organophosphate resistance in *Culex tarsalis*. J. Amer. Mosq. Control Assoc. 3:473-475.

*10. Wirth, M., Georghiou, G. P., Pasteur, N. and Luna, L. L.
1987. Evolution of resistance and change in relative density in a *Culex tarsalis* (Diptera: Culicidae) population under heavy insecticidal control. J. Med. Entomol. 24:494-497.

*11. Georghiou, G. P., Wirth, M., Tran, H., Saume, F. and Knudsen, A. B.
1987. Potential for organophosphate resistance in *Aedes aegypti* (Diptera: Culicidae) in the Caribbean area and neighboring countries. J. Med. Entomol. 24:290-294.

12. Magnin, M., Marboutin, E. and Pasteur, N.
1988. Insecticide resistance in *Culex quinquefasciatus* (Diptera: Culicidae) in west Africa. J. Med. Entomol. 25:99-104.

*13. Pasteur, N. and Georghiou, G. P.
1989. Improved filter paper test for detecting and quantifying increased esterase activity in organophosphate-resistant mosquitoes (Diptera: Culicidae). J. Econ. Entomol. (in press).

14. Ferrari, J. A. and Georghiou.
1988. Preliminary genetic studies of esterase activity variation associated with OP resistance in *Culex quinquefasciatus*. Proc. Calif Mosq. Vector Coutr. Assoc. (in press).

*15. Wirth, M. C., Marquine, M., Georghiou, G. P. and Pasteur, N.
1989. Esterases A_2 and B_2 in *Culex quinquefasciatus* Say: Role in organophosphate resistance and linkage studies. J. Med. Entomol. (submitted).

*16. Beyssat-Arnaouty, V., Mouchès, C., Georghiou, G. P. and Pasteur, N.
1989. Detection of organophosphate detoxifying esterases by dot-blot immunoassay in *Culex* mosquitoes. J. Amer. Mosq. Control Assoc. (submitted).

*17. Raymond, M., Beyssat-Arnaouty, V., Sivasubramanian, N., Mouchès, C., Georghiou, G. P. and Pasteur, N.
1989. Diversity of the amplification units of various esterases responsible for organophosphate resistance in *Culex* mosquitoes. Biochem. Genet. (submitted).

*18. Ferrari, J. A. and Georghiou, G. P.
1989. Relation of degree of gene amplification to esterase activity and insecticide resistance. Biochem. Genet. (submitted).

Table 1. Tests performed on *Culex pipiens* collections. DD = discriminating doses, BA = bioassay, EL = electrophoresis, FP = Filter paper test, and MT = Microtiter plate assay.

Localities	State or country	Year of collection	DD	BA	EL	FP	MT
NORTH AMERICA							
Anderson	California	1986	+	-	+	+	+
Butte	California	1986	+	+	+	+	+
Chico	California	1987	+	+	+	+	+
Chino	California	1988	+	+	+	+	-
Coachella	California	1985	-	-	+	-	-
Coachella	California	1987	+	-	+	+	+
Franklin	California	1987	+	-	+	-	+
Gridley	California	1987	+	+	+	+	+
Handford	California	1985	-	-	+	-	-
Long Beach (A)	California	1985	-	-	+	-	-
Long Beach (B)	California	1985	-	-	+	-	-
Long Beach (C)	California	1985	-	-	+	-	-
Los Angeles	California	1988	+	+	+	-	+
Oroville	California	1987	+	+	+	+	+
Petaluma	California	1986	+	-	+	+	+
Riverside	California	1985	-	-	+	-	-
Sacramento	California	1985	-	-	+	-	-
San Diego	California	1985	-	-	+	-	-
San Mateo	California	1987	+		+	-	-
Simpson	California	1986	+	+	+	+	+
Sutter Yuba	California	1988	+	+	+	-	-
Daytona Beach	Florida	1986	+	+	+	+	+
Naples (A)	Florida	1986	-	-	+	-	-
Naples (B)	Florida	1986	+		+	+	+
Naples (C)	Florida	1986	+	+	+	+	-
Savannah	Georgia	1986	+	+	+	+	+
Berkeley	Illinois	1986	+	+	+	+	+
Westchester	Illinois	1986	+	+	+	+	+
New Orleans	Louisiana	1986	+	+	+	+	+
New Orleans	Louisiana	1987	+	+	+	-	+
Slidell	Louisiana	1986	+	+	+	+	+
Saginaw (A)	Michigan	1987	+	+	+	+	+
Saginaw (B)	Michigan	1987	+	+	+	+	+
St Paul	Minnesota	1986			+		
Paramus (A)	New Jersey	1987	+	+	+	+	+
Paramus (B)	New Jersey	1987	+	+	+	+	+

Table 1. Continued

Localities	State or country	Year of collection	DD	BA	EL	FP	MT
Albuquerque	New Mexico	1988	+	+	+	-	-
Central Point	Oregon	1988	+	-	+	-	-
Fort Knox	Tenessee	1986	+	-	+	+	+
Memphis	Tenessee	1988	+	+	+	+	+
Houston	Texas	1986	+	+	+	+	+
Salt Lake City	Utah	1987	+	+	+	+	+
CENTRAL AMERICA							
Guatemala City	Guatemala	1987	+	+	+	+	+
Tapachula (A)	Mexico	1988	+	+	+	-	+
Tapachula (B)	Mexico	1988	+	+	+	-	+
ASIA							
Beijing	China	1988	+	+	+	-	+
Guilin	China	1988	+	+	+	-	+
Shanghai	China	1988	+	-	+	-	+
Koza	Japan	1987	+	+	+	-	+
Cheong-ju	Korea	1987	+	+	+	+	+
IRI	Korea	1987	+	+	+	+	+
Seoul	Korea	1987	+	+	+	+	+
Suweon	Korea	1987	+	+	+	+	+
Lahore	Pakistan	1985	+	+	+	+	-
Peshawar	Pakistan	1985	+	+	+	+	-
Bangkok (A)	Thailand	1987	+	-	+	-	-
Bangkok (B)	Thailand	1987	+	-	+	-	-
Bangkok (C)	Thailand	1987	+	-	+	-	-
Bangkok (D)	Thailand	1987	+	+	+	-	+
EUROPE AND MEDITERANEAN AREAS							
Moutoullas	Cyprus	1987	-	+	+	+	+
Marathon	Greece	1988	+	+	+	-	+
Cairo	Egypt	1987	-	+	+	-	-

Table 2. Adjusted strain mean log esterase activities with associated standard errors, geometric mean esterase activities with assymetric 95% confidence intervals, and strain coefficients of variation.

Exp	Strain	Sex	N	Mean Log Esterase Activity	S.E.	Geometric Mean Esterase Activity	95% Confidence Interval	Coefficient of Variation
1	S-Lab	F	48	1.041	.015	10.99	10.25 - 11.78	
		M	48	0.907	.032	8.07	6.96 - 9.36	
		X	96	0.974	.011	9.42	8.96 - 9.90	25.5
	F_1(SL)	F	67	2.899	.022	792.50	716.29 - 876.82	
		M	72	2.835	.019	683.91	626.81 - 746.22	
		X	135	2.867	.005	736.21	719.61 - 753.18	13.5
	F_1(TR)	F	47	2.879	.027	756.83	667.85 - 857.68	
		M	48	2.865	.019	732.82	671.14 - 800.18	
		X	95	2.872	.007	744.73	721.29 - 768.93	15.9
	Tem-R	F	48	3.045	.026	1109.54	983.42 - 1251.01	
		M	47	3.107	.018	1279.38	1177.12 - 1390.53	
		X	95	3.076	.007	1191.24	1153.75 - 1229.95	15.9
2	S-Lab	F	48	1.030	.010	10.72	10.23 - 11.22	
		M	49	0.977	.022	9.48	9.02 - 9.98	
		X	97	1.003	.008	10.07	9.71 - 10.44	18.2
	F_1(TR)	F	49	2.842	.017	695.02	642.46 - 751.89	
		M	49	2.792	.017	619.44	572.59 - 670.12	
		X	98	2.817	.005	656.15	641.34 - 671.30	11.5
	Tem-R	F	47	3.022	.019	1051.96	963.32 - 1148.76	
		M	49	3.071	.009	1177.61	1129.59 - 1227.67	
		X	96	3.047	.008	1114.29	1074.31 - 1155.77	18.3

Table 3. LC_{50}[a] values for the strains with 95% condfidence intervals and resistance ratios (RR[b]).

Strain	LC_{50} (mg./L.)	95% Confidence Interval	Resistance Ratio
S-Lab	0.00497	0.00448 - 0.00555	1
F_1(TR)	0.364	0.338 - 0.386	73
F_1(SL)	0.393	0.327 - 0.470	79
Tem-R	2.93	2.77 - 3.10	590

[a] LC_{50} = the concentration of insecticide expected to produce 50% mortality among the treated individuals.

[b] RR = the LC_{50} of a strain divided by the LC_{50} of the S-Lab strain

Table 4. Field populations tested by FP/Est test KIT

Code	Location	Date of Collection	Species	Control[a] (1984-88)	Larval site
LAW-1	Inglewwod CA	09.27.88	C. pipiens	Physical control	Flower containers
LAW-2	Hidden Hills CA	10.04.88	C. pipiens	Oil, PYR, TEM	Horse trough
LAW-3	LA, Hudson Av.CA	10.04.88	C. pipiens	idem	Small drain
LAW-4	Redondo Beach CA	09.27.88	C. pipiens	TEM	Small drain
LAW-5	Redondo Beach CA	09.27.88	C. pipiens	TEM	Small drain
LAW-6	LA, Thayer Av. CA	10.03.88	C. pipiens	Oil, PYR, TEM	Cement-lined channel
LAW-7	Rolling Hills Estates CA	10.04.88	C. pipiens	TEM	Small drain
LAW-8	El Segundo CA	09.08.88	C. tarsalis	Oil	large oily-water sump
NM-1	Alburquerque NM (Paradise Hill Park)	09.08.88	C. tarsalis		
NM-2	Idem	09.08.88	C. pipiens		
NM-3	Alburquerque (Coda Roberson Pl.)	09.21.88	C. pipiens		
SEMAD-1	Encino CA	08.22.88	C. pipiens	GB, Dimilin, Pirenone	water trough
SEMAD-2	Norwalk CA	09.02.88	C. pipiens	idem	water through
Coachella-1	Thermal CA	08.04.88	C. tarsalis	Oil	Okra field
Coachella-2	Indio CA	07.26.88	C. pipiens	Oil	drainage ditch
Coachella-3	Palm Desert CA	07.20.88	C. pipiens	Oil	ornemental pond
Coachella-4	Thermal CA	07.20.88	Ps. colombiae	Oil	dates
Coachella-5	Indio CA	07.14.88	C. pipiens	Oil	drainage ditch
Coachella-6	Coachella CA	07.06.88	C. pipiens	Oil	sump (fruit wash)
San Mateo-1	Foster City CA	06.27.88	C. pipiens	GB	Catch basin
San Mateo-2	Fremont CA	06.28.88	Ae. nigromaculis	TEM, GB, BTI	pasture
San Mateo-3	Sebastopol CA	07.24.88	C. tarsalis	GB	aerator pond
San Mateo-4	Novato CA	07.25.88	C. pipiens	none	tail water ditch
San Mateo-5	Santa Rosa CA	07.25.88	C. pipiens	GB	Sewage pond
Butte-1	Gridley CA	07.21.88	C. pipiens	Oil, CHL	Garbage bins
Butte-2	Chico CA	06.29.88	C. pipiens	Oil, FEN, CHL	dairy pond
Butte-4	Oroville CA	05.14.88	C. pipiens	idem	sewage pond
Butte-5	Chico CA	06.22.88	C. pipiens	Oil, FEN	dairy pond
WV-1	Simoes Dairy		C. pipiens		
WV-2	Ontario CA		C. pipiens+C. peus		
WV-3	Aquire Dairy		C. peus		
WV-4	Rocha Dairy		C. pipiens		
WV-5	Haringa Dairy		C. peus		
WV-6	Chino CA	06.17.88	C. peus		street drain
WV-7	Chino CA	06.17.88	C. pipiens		street drain
WV-8	Chino CA	06.16.88	C. peus		dairy pond
WV-9	Chino CA	06.14.88	C. peus		dairy pond
WV-10	Chino CA	06.17.88	C. peus		dairy pond

(a) PYR = pyrethrum; TEM = temephos, FEN = fenthion, CHL = chlorpyrifos.

Table 5. Comparisons of the proportions of larvae with OD<0.15 and OD<0.20 in field collections of *C. pipiens. pipiens* and *C. p. quinquefasciatus*.

Collections	% LARVAE WITH OD<0.15	OD<0.20	N(a)	X^{2}(b)
LAW-1	55	90	(20)	4.514*
" -2	100	100	(20)	0
" -3	40	40	(10)	0
" -4	55	75	(20)	0.989
" -5	55	95	(20)	6.533*
" -6	35	35	(20)	0
" -7	20	30	(20)	0.133
NM-2	93	97	(30)	0.0001
" -3	90	90	(30)	0
SEMAD-1	94	99	(105)	2.365
" -2	78	92	(75)	4.315*
COACHELLA-2	93	100	(15)	1.034
" -3	27	47	(15)	0.574
" -5	60	93	(15)	2.981
" -6	60	80	(15)	0.635
SAN MATEO-1	0	8	(50)	2.344
" -4	100	100	(24)	0
" -5	85	90	(20)	0.057
BUTTE-1	60	70	(50)	0.879
" -2	80	92	(50)	2.990
" -3	100	100	(54)	0
" -4	38	48	(50)	1.354
West V-1	100	100	(50)	0
" -2	100	100	(7)	0
" -4	82	92	(51)	1.410

(a) number of larvae tested; (b) * = significant at the 0.05 level.

Table 6. Percent mortalities observed at discriminating doses of temephos (TEM), chlorpyrifos (CHL), fenthion (FEN), malathion (MAL), propoxur (PRO) and permethrin (PER).

	% MORTALITIES					
POPULATIONS	TEM	CHL	FEN	MAL	PRO	PER
SEMAD-1	85 (80)	98* (80)	100* (80)	100* (80)	-	93 (80)
" -2	9 (80)	89 (80)	100* (80)	98* (80)	100 (80)	81 (80)
Coachella-2	60* (60)	25 (60)	48* (60)	100 (60)	100 (60)	43 (60)
" -3	18* (60)	15* (60)	10* (60)	93 (60)	100 (60)	37 (60)
" -5	23* (60)	57 (60)	15* (60)	98 (60)	98 (60)	37 (60)
" -6	57* (40)	75* (40)	10 (40)	82* (43)	69 (45)	10 (39)
San Mateo-1	25* (60)	57 (60)	23* (60)	89 (57)	100 (60)	100 (60)
" -4	100* (40)	100* (40)	100* (40)	100* (40)	100 (40)	100 (40)
" -5	100* (40)	100* (40)	80 (40)	100* (40)	100 (40)	98 (40)
Butte-1	95* (21)	100* (20)	85* (20)	100* (20)	100 (24)	81 (31)
" -2	98*(109)	96*(106)	99*(111)	99*(105)	100 (103)	48 (87)
" -3	-	100*(100)	100*(100)	100*(100)	-	-
" -4	-	63* (63)	54* (81)	32 (59)		
West V-1	100* (80)	100* (80)	100* (80)	100* (80)	100 (80)	100 (80)
" -4	100 (80)	81* (80)	74* (80)	87* (80)	100 (80)	100 (80)

* indicates mortalities not statistically different from one another at P = 0.01.

Table 7 . Geographic origin of *Culex pipiens* collections, and insensitive acetylcholinesterase (AChE-R). [+ indicates presence, - absence]

Localities	State or country	Year of collection	AChE-R
NORTH AMERICA			
Anderson	California	1986	-
Butte	California	1986	-
Chino	California	1988	-
Petaluma	California	1986	-
Simpson	California	1986	-
Sutter Yuba	California	1988	-
Daytona Beach	Florida	1986	-
Naples (B)	Florida	1986	-
Savannah	Georgia	1986	-
Berkeley	Illinois	1986	-
Westchester	Illinois	1986	-
New Orleans	Louisiana	1986	-
Slidell	Louisiana	1986	-
St Paul	Minnesota	1986	-
Paramus (A)	New Jersey	1987	-
Paramus (B)	New Jersey	1987	-
Fort Knox	Tenessee	1986	-
Houston	Texas	1986	-
CENTRAL AMERICA			
Guatemala City	Guatemala	1987	-
Tapachula (A)	Mexico	1988	-
Tapachula (B)	Mexico	1988	-
ASIA			
Beijing	China	1988	-
Guilin	China	1988	-
Shanghai	China	1988	-
Koza	Japan	1987	-
Cheong-ju	Korea	1987	-
IRI	Korea	1987	-
Seoul	Korea	1987	-
Suweon	Korea	1987	-
Thailand (D)	Thailand	1987	-
EUROPE AND MEDITERANEAN AREAS			
Moutoullas	Cyprus	1987	+
Marathon	Greece	1988	+

Fig. 1. Relationship between adult age and non-specific esterase activity in *C. quinquefasciatus*. (Tested in two series: Ser I., males only; Ser. II, males and females).

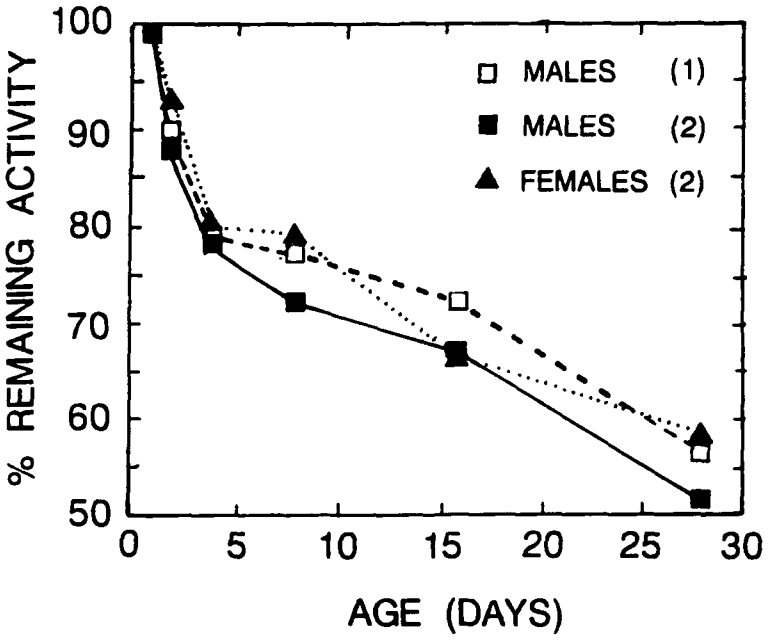


Fig. 2. Changes in level of non-specific esterase activity in *C. quinquefasciatus* adults following 1-hr exposure to fenitrothion.

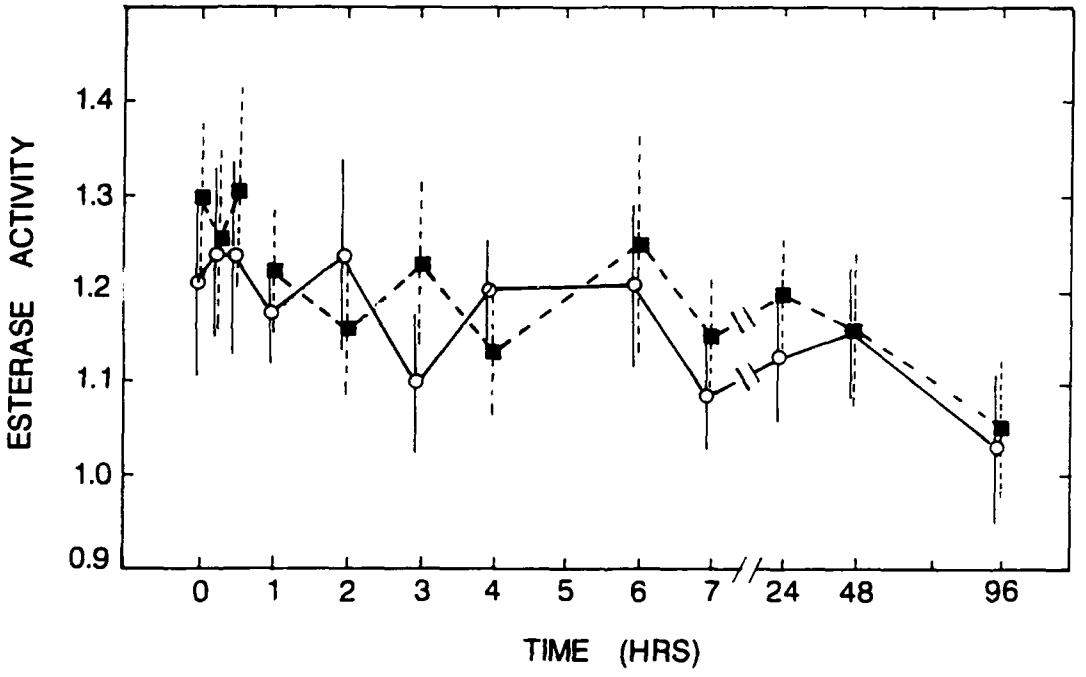


Fig. 3. *In vitro* inhibition of non-specific esterase activity of homogenates of *C. quinquefasciatus* by various concentrationsof fenitrothion.

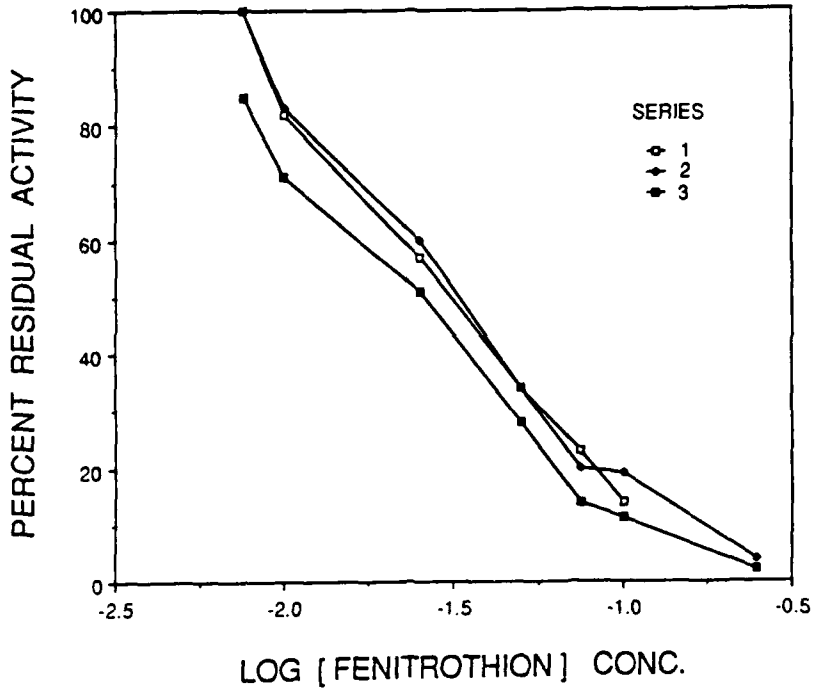


Fig. 4. Frequency distributions of log esterase activities (nmoles alpha-naphthyl hydrolyzed/min.) for the Tem-R and F_1 strains produced in experiment 1.

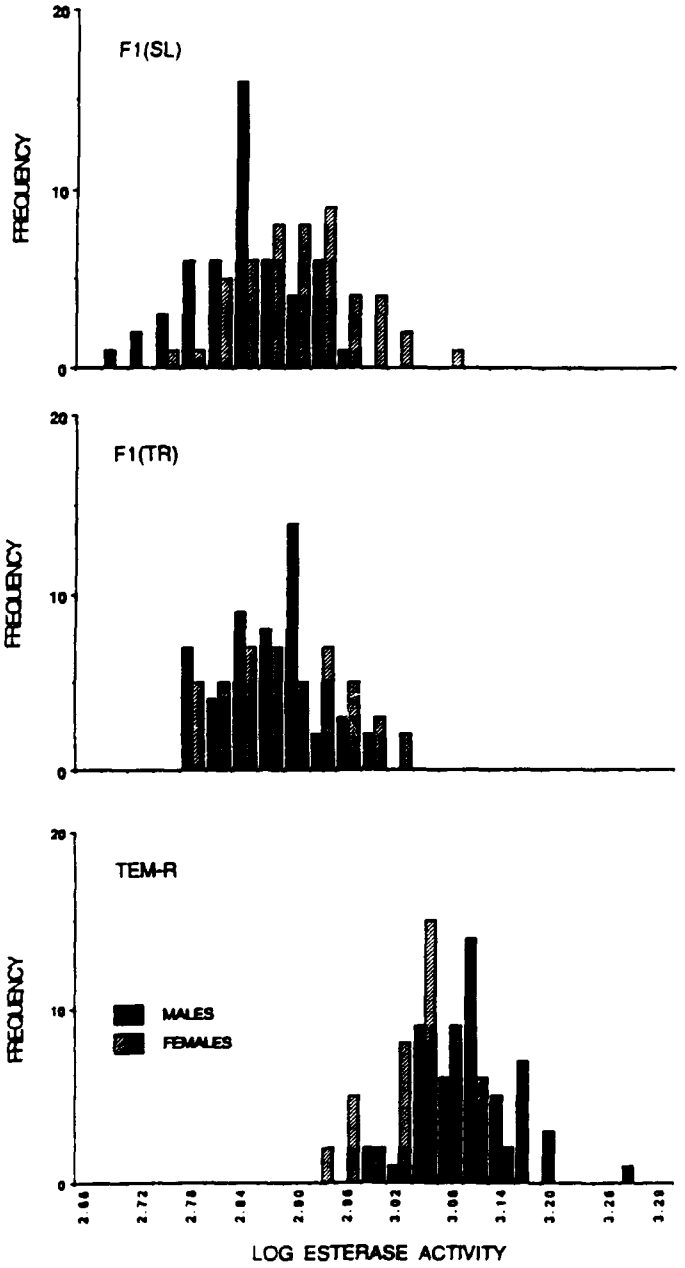


Fig. 5. Frequency distributions of log esterase activities (nmoles alpha-naphthyl acetate hydrolyzed/min.) for the Tem-R and F_1 (TR) strains produced in experiment 2.

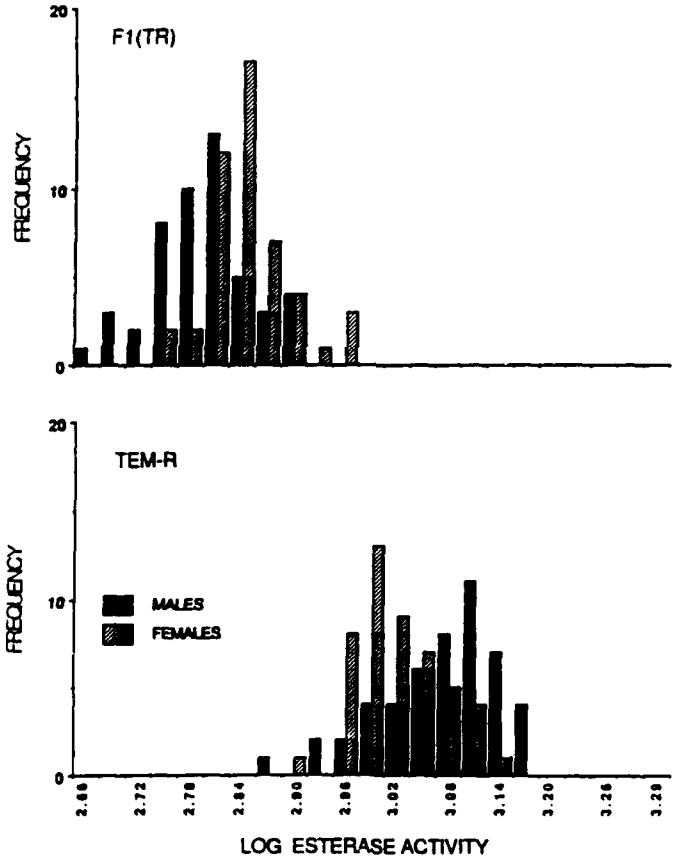


Fig. 6. Frequency distributions of log esterase activities (nmoles) alpha-naphthyl acetate hydrolyzed/min.) for the S-Lab strains produced in experiments 1 and 2.

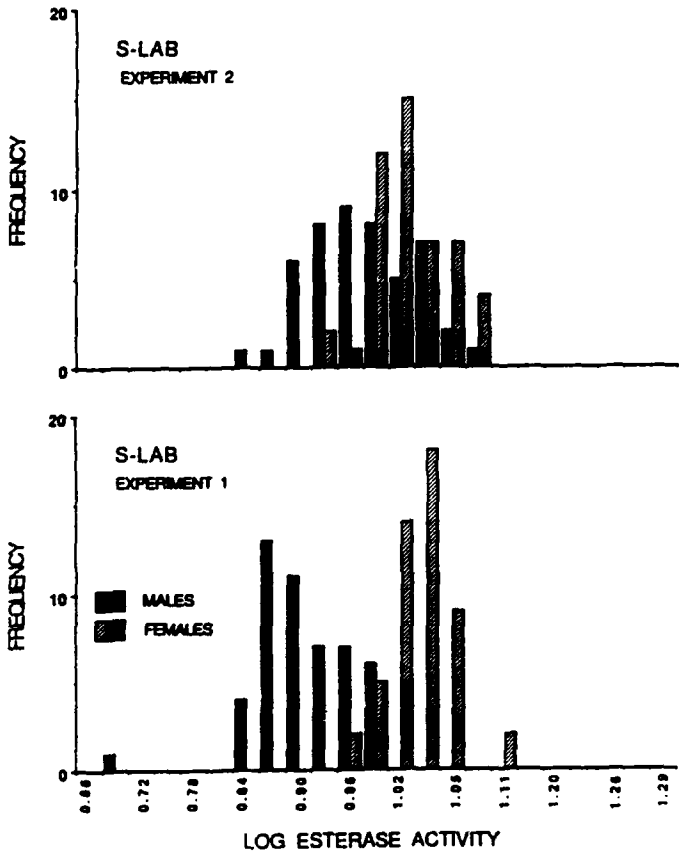


Fig. 7. Log-dose mortality lines for temephos determined for the A line each generation.

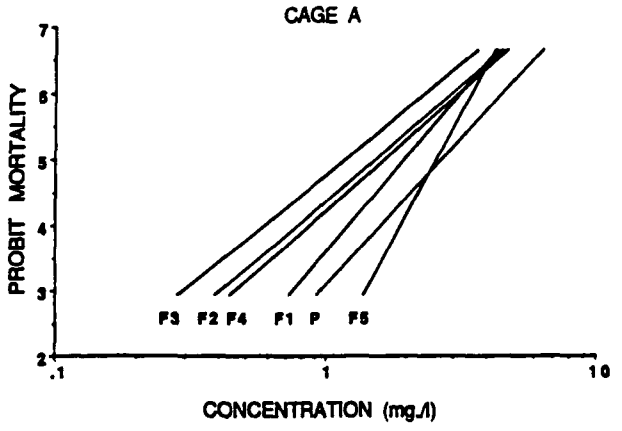


Fig. 8. Log-dose probit mortality lines for temephos determined for the B line each generation.

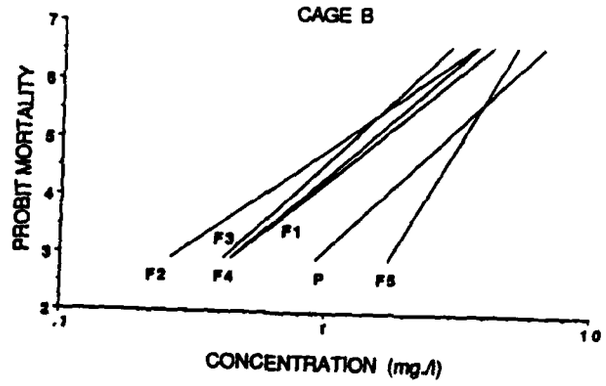


Fig. 9. The mean (horizontal bar) and range (vertical bar) of esterase activity determined for the A line each generation.

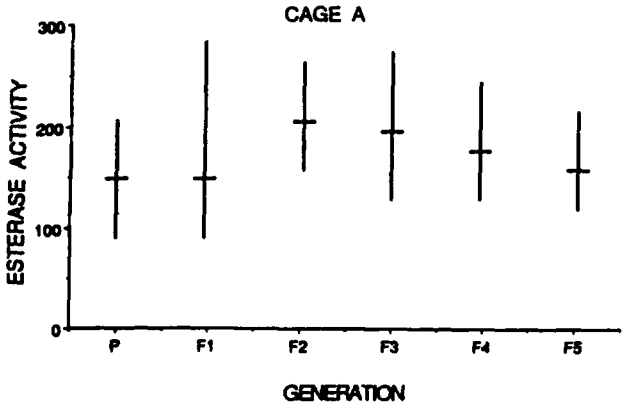


Fig. 10. The mean (horizontal bar) and range (vertical bar) of esterase activity determined for the B line each generation.

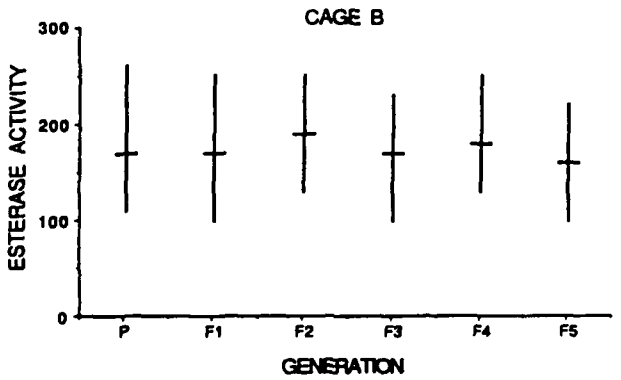


Fig. 11. Distribution of mosquitoes of three field collections of *C. pipiens* according to optical density of filter paper test spots. Dark bars indicate mosquitoes classified as resistant, open bars, as susceptible, based on visual interpretation of test spots by field entomologists.

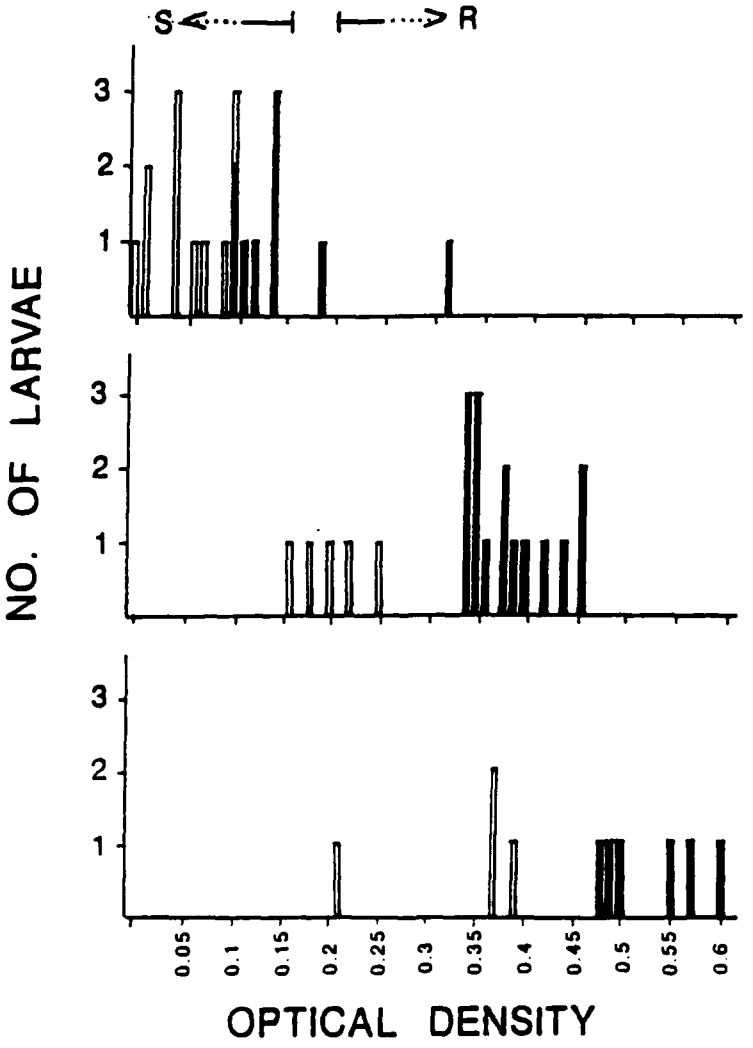


Fig. 12. Effect of length of post-homogenization period on esterase activity (optical density) determined by filter paper test.

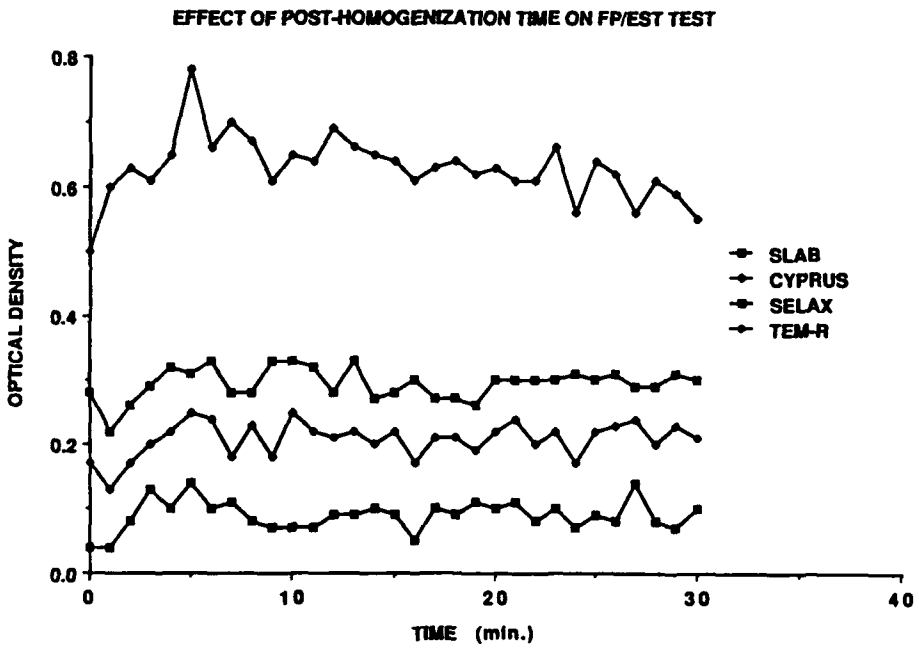


Fig. 13. Effect of length of drying time of mosquito homogenate deposit on filter paper on esterase activity (optical density).

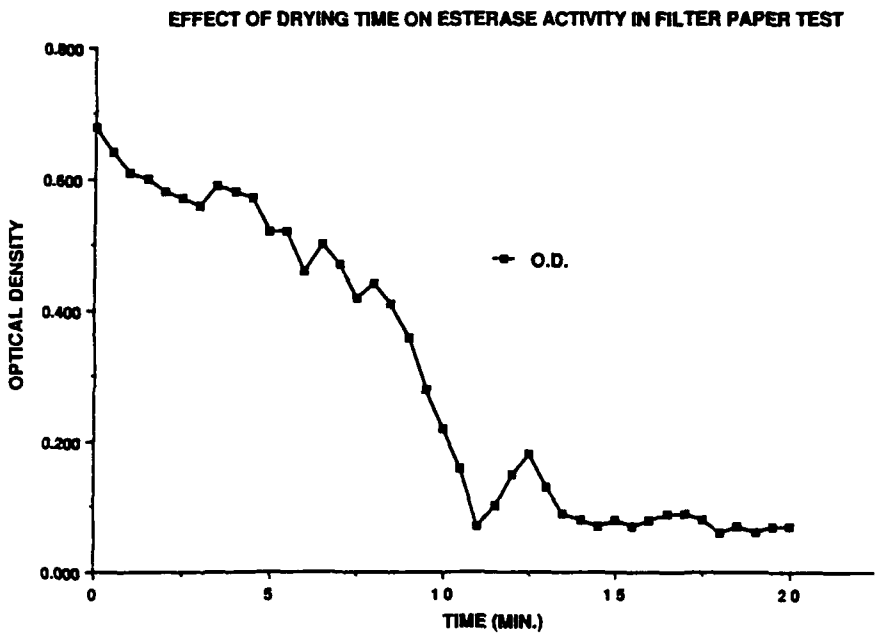
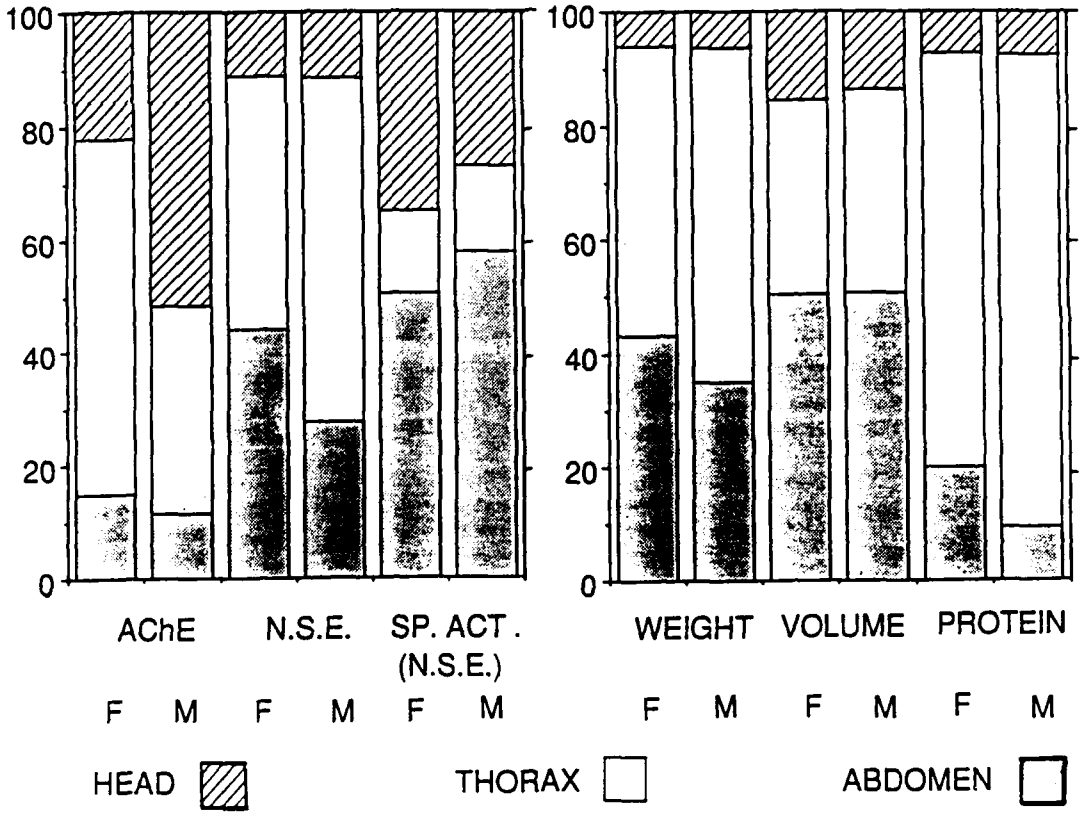


Fig. 14. Distribution of non-specific esterase (NSE) and acetylcholinesterase (AChE) activity in head, thorax and abdomen of *C. quinquefasciatus*. (F = female; M = male; Sp. Act. = specific activity).



UNIVERSITY OF CALIFORNIA, RIVERSIDE

BERKELEY • DAVIS • IRVINE • LOS ANGELES • RIVERSIDE • SAN DIEGO • SAN FRANCISCO SANTA BARBARA • SANTA CRUZ

COLLEGE OF NATURAL AND
AGRICULTURAL SCIENCES
DEPARTMENT OF ENTOMOLOGY
5419 WEBBER HALL EAST

RIVERSIDE, CALIFORNIA 92521

W.H.O. COLLABORATING LABORATORY
FOR RESEARCH ON INSECTICIDE RESISTANCE

November 29, 1988.

FIELD KIT FOR DETECTION OR ESTERASES INVOLVED IN ORGANOPHOSPHATE RESISTANCE IN MOSQUITOES (FP/EST TEST)

A major mechanism of resistance to organophosphate insecticides in mosquitoes is detoxification by esterase enzymes. In addition to degrading organophosphate insecticides, these esterases are also able to degrade *alpha*- and *beta*-naphthyl acetate, and these chemicals may be used as working substrates in order to detect the presence of detoxifying esterases and hence of resistance.

We have earlier described a simple "filter paper test" for detecting increased esterase activity in single mosquitoes (N. Pasteur and G. P. Georghiou, 1981, Mosquito News 41: 181-183). The test is based on the deposition of mosquito homogenates on filter paper which is then incubated in buffer containing *a*-naphthyl acetate and then in a staining solution containing an azodye. The test enables the unambiguous discrimination between susceptible and organophosphate-resistant insects. The test was recently modified and improved (N. Pasteur and G. P. Georghiou, J. Econ. Entomol. in press) and a practical "test kit" has been derived from it. This kit, described in the following pages, is intended for use by field personnel for the detection and monitoring of the frequency of organophosphate-susceptible or -resistant individuals in laboratory or field populations of mosquitoes. The kit has been used extensively on mosquitoes of the *Culex pipiens* complex.

We request your collaboration in testing this kit on mosquitoes of your own area. Initially, the kit should be tested in parallel with bioassay utilizing the diagnostic dose for resistance that is appropriate for the species involved, as prescribed by WHO (WHO Tech. Rept. Ser. No. 737, p. 86, 1986). We would appreciate your returning to us the enclosed report forms together with your comments or questions.

Thank you.

George P. Georghiou
Professor of Entomology

*FIELD KIT FOR DETECTION OF ESTERASES INVOLVED IN ORGANOPHOSPHATE RESISTANCE IN CULEX MOSQUITOES (FP/EST test)**

KIT CONTENTS

1. One plastic sealable box
2. 5 g Na_2HPO_4 and 8 g NaH_2PO_4 salts for substrate and homogenization buffers (Sigma-0751 and Sigma-0876)
3. 2 g *alpha*-naphthyl acetate (Sigma, N8505)
4. 3 g Fast Garnett GBC salt (Sigma F0875)
5. 0.5 ml Triton X-100
6. Small funnel
7. 96-well microtiter plate (Fisher-14245145)
8. 2 homogenization rods (1 + spare)
9. Insect forceps
10. 5 pipette tips (Fisher 22-35-130-3)
11. 2 calibrated 1 ml disposable transfer pipettes (318-001-PGC)
12. 2 calibrated microtubes. Each tube is calibrated (see etched line) for volume of each salt needed to make 100 ml buffer.
13. 50 Whatman No. 2 filter paper strips (Fisher 09-815A)
14. Spatula to measure Fast Garnett GBC salt.

Optional:

Porcelain plates with 12 cavities (Fisher, 13-745) in lieu of microtiter plate.

Automatic pipette, dispensing 2 to 200 µl volumes.

*Attention: When not in use, test kit must be kept in refrigeration or freezer.

PREPARATION OF BUFFERS AND SOLUTIONS

1. Substrate Buffer (Buffer A).

   - To prepare 1 liter of buffer A, place entire quantity of two sodium salts provided (item 2) into 1 liter of water (preferably distilled). This will produce 0.1 M sodium phosphate buffer (A) at pH 6.5. Label as BUFFER A. Keep refrigerated. May be used over several weeks.

   - If a smaller volume of buffer is desired, you may prepare 100 ml (* see footnote) by using the two salts at the volumes indicated by the lines etched on each microtube. (Observe labels identifying each tube). Tap microtubes to compress salt crystals.

2. Homogenization Buffer (Buffer B)

   - Prepare 100 ml of Buffer A, as above, or use 100 ml from the larger volume you have prepared.
   - Add the 0.5 ml Triton X-100 .
   - Label as Buffer B.
   - Keep refrigerated when not in use.

3. SUBSTRATE SOLUTION (Solution C)

   - Dissolve the 2 g *alpha*-naphthyl acetate in 100 ml ethanol (ETOH). Label as SOLUTION C.
   - Keep in freezer (-20C) when not in use.

TEST SET UP

1. Measure 100 ml of buffer (A) into a bowl. (Label as bowl 1.)
   To this bowl add 5 ml of substrate solution (C) using the 1 ml transfer pipette 5 times. Rinse the pipette.

2. Into a second bowl (bowl 2), measure 100 ml of clean water.
   To the water add a scoopful of Fast Garnet GBC salt using the marked tip of the spatula provided. Stir to dissolve.

---

* If a graduated cylinder is not available, note that the test kit box can be used for this purpose: hold the box in a tilted position and add water up to the two (black) lines indicated. This is the desired volume.

TEST PROCEDURE

1. Place mosquitoes (larvae or adults) singly in wells of microplate. Do not use more than 15 mosquitoes in any single test. If adults are to be used these should be kept alive until ready for testing.

2. Add 100 ul homogenization buffer (B) to each well. To measure 100 ul, count 4 drops of the buffer using the transfer pipette provided.

3. Grind each mosquito (about 10 sec.) with the plexiglass rod provided. After each homogenization, rod must be dipped in a glass of water and carefully wiped with tissue paper to avoid contamination between samples.

4. Transfer 2 ul of each homogenate onto the filter paper as follows: hold the filter paper from one corner with the index finger and thumb; immerse the yellow pipette tip to the bottom of the well; place your finger over the top of the pipette to retain the fluid in it and spot the sample lightly on the filter paper. To completely expel the fluid, press the top of the pipette gently with finger. A uniform spot should be produced. Clean the pipette tip after each transfer by dipping it in water and bring it in contact with tissue paper.

5. After processing the samples (approx. 2-3 min., but not longer than 5 min.), immerse the filter paper in substrate solution (bowl 1) for 60 seconds.

6. Using the forceps, remove the filter paper and blot it gently between two layers of paper toweling.

7. Immerse the filter paper in the staining solution (bowl 2) for 60 seconds.

8. Remove the filter paper and repeat the blotting step (No. 6).

9. Rinse paper by immersing briefly (2 sec.) in water and air dry on paper towel.

10. Examine and record the results: The presence of esterase is revealed by the development of a purple color at the site of each deposit. Homogenates of organophosphate-susceptible mosquitoes stain faintly purple whereas those of organophosphate-resistant mosquitoes stain more strongly.

11. Differences in staining intensity are evident by eye. If quantitative readings are desired, they may be obtained on a densitometer (such as Tobias Associates, Inc., Ivyland, PA, model RCX) using a red filter.

12. Dried filter papers may be stored indefinitely in a note book, or in a plastic bag in the dark, for future reference.

Further reading:

Georghiou, G. P. and N. Pasteur. 1978. Electrophoretic esterase patterns in insecticide-resistant and -susceptible mosquitoes. J. Econ. Entomol. 71:201-205.

Pasteur, N. and G. P. Georghiou. 1981. Filter paper test for rapid determination of phenotypes with high esterase activity in organophosphate resistant mosquitoes. Mosq. News 41:181-183.

Pasteur, N. and G. P. Georghiou. 1988. Improved filter paper test for detecting and quantifying increased esterase activity in organophosphate-resistant mosquitoes. J. Econ. Entomol. (in press).

Raymond, M., N. Pasteur, G. P. Georghiou, R. B. Mellon, M. C. Wirth, and M. K. Hawley. 1987. Detoxification esterases new to California, USA, in organophosphate-resistant *Culex quinquefasciatus* (Diptera:Culicidae). J. Med. Entomol. 24:24-27.

Fur further information contact:

Dr. George P. Georghiou
Department of Entomology
University of California
Riverside, CA 92521
Tel: (714) 787-5830

or

Dr. Nicole Pasteur
Laboratoire de Genetique
Institut des Sciences de l'Evolution
Universite de Montpellier
34060 Montpellier, France
Tel: (33) 67-61-06-40

Diagnostic dose test

For the purpose of standardization, we recommend that you test a total of 60 to 100 early 4th-instar larvae with each insecticide, using 20 larvae per beaker (containing 99 ml water and 1 ml of insecticide standard solution). It is suggested that these tests be spread over two or more days. Each collection should be tested with both the diagnostic dose test and the FP/Est test (see below).

Filter paper test

We recommend that you test 50 4th-instar larvae by this method, using 10 to 15 larvae in each run. Young adults may be used instead of larvae. All FP/Est tests may be done on the same day.

The preferred species for these tests are *Culex quinquefasciatus* and *C. pipiens*. However, other species of *Culex*, as well as species of *Anopheles* or *Aedes*, may also be tested. For each collection please indicate the particulars requested and your results on the appropriate report form.

Please number your filter papers in sequence, indicating the initials of your District in front of the collection number.

Please mail to:
G. P. Georghiou
W.H.O. Collaborating Center for
Research on Insecticide Resistance
Department of Entomology
University of California,
Riverside, CA 92521

MOSQUITO COLLECTION RECORD

Species:

Date of Collection:

Location (nearest town):

Larval habitat (dairy effluent, pond, swimming pool, drainage ditch, etc.):

Life stage collected:

Generation sent (field, F_1, F_2, etc.):

Mosquito population density (briefly):

Notes on chemical control:

1981-82:

1983-84:

1985-86:

1987-88:

Is resistance suspected?

Collector: **Name:**
Address:

Telephone:

Please mail to:
G. P. Georghiou
W.H.O. Collaborating Center for
Research on Insecticide Resistance
Department of Entomology
University of California
Riverside, CA 92521

DIAGNOSTIC DOSE TESTS

Species _____ **Investigator** _____

Strain/Collection _____ **Address** _____

Test date / Test No. / Insecticide (% cons.)*	Results of replications No. dead/No. tested					Σ/n	% Dead

*(See attached tables from WHO Tech. Rept. Ser. 737, p. 86, 1986)

Comments

FILTER PAPER TEST

SPECIES: STRAIN

TYPE OF TEST: FP/Est _____ FP/AChE _____ (check one)

Controls: I _____ II _____

TEST DATE: Mo. _____ Day _____ Year _____

Insect No. Stage (L,A)*	1	2	3	4	5	6	7	8	9	10	Controls I	Controls II
Visual categ. (S,R)*												
Densitometer reading												

SUMMARY: No. tested _____ No. R _____ % R _____

COMMENTS:

PAPER MOUNT

* L = larvae; A = adult; S = susceptible; R = resistant